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A Focused Microarray to Assess Dopaminergic and Glial Cell Differentiation from Fetal Tissue or Embryonic Stem Cells

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ABSTRACT

We designed oligonucleotide gene-specific probes to develop a focused array that can be used to discriminate between neural phenotypes, identify biomarkers, and provide an overview of the process of dopaminergic neuron and glial differentiation. We have arrayed approximately 100 genes expressed in dopaminergic neurons, oligodendrocytes, and astrocytes, an additional 200 known cytokines, chemokines, and their respective receptors, as well as markers for pluripotent and progenitor cells. The gene-specific 60-mer 3′ biased oligonucleotides for these 281 genes were arrayed in a 25 × 12 format based on function. Using human adult brain substantia nigra, human embryonic stem cells (ESCs), and the differentiated progeny of pluripotent cells, we showed that this array was capable of distinguishing dopaminergic neurons, glial cells, and pluripotent cells by their gene expression profiles in a concentration-dependent manner. Using linear correlation coefficients of input RNA with output intensity, we identified a list of genes that can serve as reporting genes for detecting dopaminergic neurons, glial cells, and contaminating ESCs and progenitors. Finally, we monitored NTera2 differentiation toward dopaminergic neurons and have shown the ability of this array to distinguish stages of differentiation and provide important clues to factors regulating differentiation, the degree of contaminating populations, and stage of cell maturity. We suggest that this focused array will serve as a useful complement to other large-scale arrays in routine assessment of cell properties prior to their therapeutic use. STEM CELLS *2006;24:865–875*

INTRODUCTION

The adult brain has a limited capacity to regenerate new neurons, particularly in the substantia niagra, spinal cord, and cortex (reviewed in [1]). In contrast, glial cells can be regenerated at low numbers throughout the adult life span and the numbers generated increase substantially after injury (reviewed in [2]). Despite the ability of the adult brain to undergo neurogenesis and glial genesis, a deficiency in adequate neural replacement after cell death by disease or injury results in a variety of debilitating neurological conditions. In particular, neurological disorders associated with loss of specific neural cell types, such as oligodenocyctes or dopaminergic neurons, have led to a search for disease treatments, including mobilization of endogenous stem/precursor cells to generate suitable replacement cells, development of methods to deliver or induce secretion of trophic molecules to prevent cell loss, and transplantation of cells for localized repair [3–6].

Multiple classes of cells have been considered for cell therapy, including neural stem cells (NSCs), glial restricted precursor cells, embryonic stem cells (ESCs), mesenchymal stem cells, and transdifferentiated cells [7, 8]. In each case, questions concerning the character of the transplanted population, signals directing differentiation, and specificity of differentiation have been raised. Addressing these issues has been difficult due to the lack of consensus over surrogate markers of efficacy and the availability and cost associated with assessing

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a reasonable number of markers. Multiple analytical techniques to assess gene expression in defined cell types have been developed, including microarray analysis, EST (expressed sequence tag) enumeration, SAGE (serial analysis of gene expression), and MPSS (massively parallel signature sequencing). Focused microarray analysis offers an advantage by allowing one to develop a customized array containing genes specific to particular cell populations or signaling pathways. Recently, focused microarrays have been designed to assess the state of ESC differentiation and characteristics of the undifferentiated ESC state [9, 10]. Application of these arrays has allowed researchers to evaluate the state of cells in a relatively inexpensive, rapid, and reliable way. We reasoned that a focused array encompassing a substantial, but not overwhelming, number of genetic markers for oligodendrocytes and dopaminergic neurons would allow rapid assessment of the state of a cell population prior to their use in cell replacement therapy and would be as good as other, more expensive large-scale array methods.

We have developed a focused array of approximately 280 genes that includes markers for dopaminergic neurons, glia, neural progenitors, pluripotent cells, and signaling molecules thought to be important in regulating neural differentiation. Additionally, we included cytokines, chemokines, and their receptors to provide a global view of the potential signaling pathways that may regulate the process of differentiation. Using human adult brain substantia nigra, human ESC (hESC)–derived NSCs, and pluripotent hESCs, we show that the array is capable of distinguishing these populations by their gene expression profiles. Moreover, we have used this array to monitor NTera2 differentiation into dopaminergic neurons. We show that such an array can monitor the process of differentiation and provide important clues to factors that regulate the differentiation process.

Materials and Methods

Construction of a Focused Array

Based on a literature research and our understanding of neural development, a list of candidate genes (Table 1) with the National Center for Biotechnology Information (NCBI; Bethesda, MD, http://www.ncbi.nih.gov) Refseq number was prepared. The focused array used one gene-specific 60-mer 3′ biased oligonucleotide for each gene. The probes were designed through a rigorous design methodology that was developed by SuperArray Bioscience Corporation (Frederick, MD, http://www.superarray.com). Briefly, candidate probes were selected starting from the 3′ end of transcripts based on criteria such as melting temperature (T_m) and guanine-cytosine (GC) content. These 60-mer sequences were then subjected to a BLAST (basic local alignment search tool) search screening against all known transcripts in GenBank to eliminate crossreactive sequences. The candidate probes that were likely to cross-hybridize with other nonself sequences were further eliminated. This process would continue selecting sequence from the 3′ end of a transcript until an optimal probe was identified. In general, most of the probes were designed within 1,000 base from 3′ end and had a T_m of close to 92°C and a GC content of approximately 49%. These 60-mer probes were then synthesized by Qiagen (Valencia, CA, http://www1.qiagen.com). Lyophilized oligonucleotide probe samples were suspended and adjusted to 0.1–10 μM in deionized water with phenol red (0.001%) as the tracking dye to monitor the array printing quality. A Cartesian SynQuad Prosys dispensor (Genomic Solutions, Ann Arbor, MI, http://www.genomicsolutions.com) was used to dispense between 10 and 15 nl of oligo DNA solution onto nylon membranes (Biodyne B; Pall Bioscience, East Hill, NY, http://www.pall.com). All array spots were arranged in a rectangular area (23 × 35 mm). Spot diameter was between 0.6 and 0.8 mm. The spot-to-spot distance was 1.125 mm. The printed membrane was air-dried at room temperature overnight and then subjected to 1,200 J UV crosslinking. The array was stored at −20°C until used.

A series of human housekeeping genes with varying expression levels in cells were included in each array as positive control. Biotin-labeled artificial DNA probes were also printed on microarray as detection controls. Additional control features such as bacterial probes, rRNA probes, and artificial sequences were also printed on the array.

Cultures of hESCs and NTera2 Cells

hESC lines BG01 and BG03 were obtained from BresaGen (Thebarton, SA, Australia) and grown using published protocols described elsewhere [11]. Briefly, hESCs (20,000 cells/cm^2) were cultured on inactivated mouse embryonic fibroblast (MEF) feeder cells in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12; 1:1) supplemented with 20% knockout serum replacement, 2 mM nonessential amino acids, 2 mM L-glutamine, 50 μg/ml Penn-Strep (all from Invitrogen, Carlsbad, CA, http://www.invitrogen.com), 0.1 mM β-mercaptoethanol (Specialty Media, Phillipsburg, NJ, http://www.specialtymedia.com), and 4 ng/ml of basic fibroblast growth factor (bFGF; Sigma, St. Louis, http://www.sigmaaldrich.com). After 4–5 days, cells were then dissociated by using Cell Dissociation Buffer (Invitrogen) and passaged on mitotically inactivated MEF at a density of 20,000 cells per cm^2. The culture medium was changed every other day. Under these conditions, these cells remained Oct3/4- and SSEA4-positive undifferentiated stem cell status [11].

The NTera2 cell line was obtained from American Type Culture Collection (Manassas, VA, http://www.atcc.org) and cultured according to the manufacturer's protocol with minor modifications. Briefly, cells were plated at a density of 10,000–15,000 cells per cm^2 at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 50 μg/ml penicillin/streptomycin (all from Invitrogen). The media was changed every 2–3 days until cells become confluent. The cells were either passaged using a Costar cell scraper (Krackeler Scientific Inc., Albany, NY, http://www.krackeler.com) or used for RNA extraction.

Derivation and Cultures of NSC

NSCs used were derived from the BG03 hESC line. Briefly, after 1 week of culture on mouse feeder cell layer, hESCs were fed every other day with derivation medium containing DMEM/F-12 medium (1:1; Gibco, Grand Island, NY, http://www.invitrogen.com) supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1X N2 (Gibco), and 4 ng/ml of basic fibroblast growth factor (Sigma) for 7 days. The mouse feeder layer was then removed physically using a pipette, allowing neuroepithelial precursor to attach to the culture dish and develop rosettes after 3 days in derivation medium [12].

Table 1. Gene list for the dopaminergic and glial cell focused array

Groups	No. of genes	Gene name								
Dopaminergic markers	34	*DBH*	*DRD1IP*	*DRD5*	*GFRA2*	*MAOA*	*PITX3*	*SLC6A3*	*SULT1A1*	*TPH1*
		DCT	*DRD2*	*EN1*	*GFRA3*	*MAOB*	*SLC18A2*	*SMO*	*SULT1B1*	*TPH2*
		DDC	*DRD3*	*GCH1*	*GFRA4*	*MOXD1*	*SLC18A3*	*SNCAIP*	*SULT1C1*	
		DRD1	*DRD4*	*GFRA1*	*LMX1B*	*NR4A2*	*SLC6A2*	*SNCB*	*TH*	
Glial markers	93	*ALS2*	*CTNS*	*GJB1*	*GRINL1A*	*Kir1.2*	*NG2*	*PDGFRB*	*SLC1A3*	*TRPC3*
		ARSA	*CTSB*	*GLB1*	*GRM2*	*HES5*	*NKX2–2*	*PLP1*	*SLC1A6*	*TUBA1*
		ARSB	*CTSC*	*GNPTG*	*GRM7*	*BRG1*	*NKX2–5*	*POU3F1*	*SLC1A7*	*UGT8*
		ASAH1	*CTSD*	*GRIA1*	*HES1*	*MAG*	*NKX6–1*	*POU4F2*	*SMARCA4*	*ZNF180*
		BCAN	*CTSL*	*GRIA4*	*HEXA*	*MBP*	*NKX6–2*	*PPT1*	*SMPD1*	*ZNF235*
		BIRC5	*EGR1*	*GRID1*	*HEXB*	*MCOL*	N1	*NPC1*	*PSAP*	*SMPD2*
		CD44	*EGR2*	*GRIK1*	*ID1*	*MCOLN2*	*NPC2*	*S100B*	*SOX11*	
		CLN3	*GAL3ST1*	*GRIK2*	*ID2*	*MCOLN3*	*OLIG1*	*SIAT8A*	*SOX4*	
		CSPG3	*GCM2*	*GRIN1*	*ID3*	*MOG*	*OLIG2*	*SLC17A8*	*SULF1*	
		CST3	*GDNF*	*GRIN2B*	*ID4*	*MPZL1*	*PAX3*	*SLC1A1*	*TAPBP*	
		CSTB	*GFAP*	*GRINA*	*KCNA4*	*MYT1*	*PDGFRA*	*SLC1A2*	*TNC*	
Contaminating embryonic stem cells and progenitors	36	*ABCG2*	*Dppa5*	*NANOG*	*NGFR*	*POU3F2*	*SOX1*	*TERF1*	*VIM*	
		ACTA2	*MAP2*	*NCAM1*	*NTRK2*	*POU3F3*	*SOX2*	*TERT*		
		ACTG2	*MCM2*	*NEF3*	*NTRK3*	*POU5F1*	*SOX3*	*TINF2*		
		CER1	*MSI1*	*NEFL*	*PODXL*	*PROM1*	*SYT1*	*VCAM1*		
		DCX	*MSI2*	*NES*	*PODXL2*	*SLC2A1*	*TEP1*	*VEGF*		
Signaling and others	118	*ACVRL1*	*CDH4*	*EGFR*	*FZD2*	*IGF1R*	*KDR*	*PDE1B*	*TGFB1*	
		ALDH1A1	*CDH5*	*ERBB2*	*FZD3*	*IGF2*	*KLF16*	*PDGFA*	*TGFB2*	
		BDNF	*CHAT*	*ERBB3*	*FZD4*	*IGF2R*	*KV3.1*	*PDGFB*	*TGFB3*	
		BMP1	*CNTF*	*ERBB4*	*FZD7*	*IL6R*	*LIF*	*PTCH*	*TGFBR1*	
		BMP15	*CNTFR*	*FGF1*	*FZD8*	*IL6ST*	*LIFR*	*PTCH2*	*TGFBR2*	
		BMP2	*CTNNB1*	*FGF17*	*FZD9*	*INHBA*	*Neuregulin 4*	*PTEN*	*TGFBR3*	
		BMP3	*CXCL12*	*FGF2*	*GAD1*	*INHBB*	*NOTCH1*	*QDPR*		
		BMP4	*CXCR4*	*FGF4*	*GDF1*	*INSRR*	*NOTCH2*	*RB1*		
		BMPR1A	*DLK1*	*FGF5*	*GDF3*	*ISL1*	*NOTCH3*	*RBL1*		
		BMPR1B	*DNMT1*	*FGF9*	*GDF8*	*ITGA4*	*NRG1*	*RBL2*		
		BMPR2	*DNMT2*	*FGFR1*	*GDF9*	*ITGA5*	*NRG2*	*SHH*		
		CCK	*DNMT3A*	*FGFR2*	*GLS*	*ITGA6*	*NTF3*	*SNAI1*		
		CCKAR	*EDN1*	*FGFR3*	*HMP19*	*ITGAV*	*NUMB*	*SNAI2*		
		CCKBR	*EDN2*	*FGFR4*	*HSPCB*	*ITGAX*	*PACAP*	*SOD1*		
		CDH1	*EDN3*	*FOXG1A*	*IFNAR2*	*ITGB1*	*PAX6*	*SOX10*		
		CDH2	*EGF*	*FZD1*	*IGF1*	*ITGB4*	*PCBD*	*SOX6*		
Positive detection control	1	*BAS2C*								
Housekeeping	2	*ACTB*	*GAPD*							
Positive hybridization controls	2	*B2M*	*RPS27A*							
Negative hybridization controls	5	*AS1*	*AS1R1*	*AS1R2*	*Blank*	*PUC18*				

Derived NSCs were propagated further on 20 μg/ml of polyornithine and 1 μg/ml of laminin-coated dishes in neurobasal medium (Gibco) supplemented with 2 mM L glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1 X B27 (Gibco), 20 ng/ml bFGF (Sigma), and 10 ng/ml of leukemia inhibitory factor (Chemicon, Temecula, CA, http://www.chemicon.com). The culture medium was changed every other day, and cells were then passaged by mechanical triturating or by trypsin when confluent. The NSCs used in this paper had undergone four passages and were uniformly Oct3/4- and SSEA4-negative and Nestin- and Sox2-positive.

Preparation of PA6-Induced PSA-NCAM–Sorted Differentiated NTera2 Cells

Neural differentiation of NTera2 cells was induced using the mouse stromal cell line PA6 as described by Schwartz et al. with minor modifications [13]. Briefly, PA6 cells were grown and maintained in minimum essential medium-α supplemented with 10% FBS and 50 U/ml penicillin/streptomycin (all from Invitrogen) until confluent. NTera2 cells were then seeded onto this confluent monolayer of PA6 feeder cells at a density of 2,000 cells per cm^2. The day at which NTera2 cells were seeded onto PA6 cells was taken to be day 1. After 12 days in culture, these NTera2-PA6 co-cultures were used to isolate polysialic acid (PSA)-NCAM–positive cells by flow cytometry sorting as previously described [13]. These PSA-NCAM–positive cells were then used for total RNA extraction and subsequent analysis.

RNA Preparation, Reverse Transcription–Polymerase Chain Reaction Amplification, and Focused Oligo-Microarray Analysis

Total RNAs from indicated cells were isolated using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, http://www.isotexdiagnostics.com) by following the manufacturer's instructions. Total RNA derived from adult human substantia nigra was purchased from Clontech (Palo Alto, CA, http://www.clontech.com). The cDNAs were synthesized using a Superscript II reverse transcriptase kit with 1 μg total RNA and 500 ng oligo(dT)12–18 (both from Invitrogen). The polymerase chain reaction (PCR) was performed in a 20 μl reaction solution containing 2 μl 10 $\times$ PCR buffer, 150 nmol $MgCl_2$, 10 nmol dNTP, 20 pmol primer, 1 μl 50x diluted cDNA, and 1 U RedTag

DNA polymerase (Sigma). The PCRs were run as follows: 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, and final extension for 10 minutes at 72°C. Primer sequences are shown in Table 2.

Focused oligo-microarrays (SuperArray Bioscience Corporation) were used for microarray analysis. Biotin-labeled cRNAs, known as labeled cRNA targets, were generated using TrueLabeling-AMP Linear RNA Amplification Kit following manufacturer's protocol (SuperArray Bioscience Corporation). Briefly, total RNA (3 μg/array) was first converted to cDNA at 42°C for 50 minutes. These cDNAs were then in vitro transcribed to cRNAs in the presence of biotin-16-UTP (Roche Molecular Biochemicals, Basel, Switzerland, http://www.roche.com). Biotin-labeled cRNAs were purified using a RNeasy Mini Kit (Qiagen). The concentration of cRNAs was measured with a UV spectrophotometer (Amersham, Piscataway, NJ, http://www.amersham.com). The array filters were hybridized with these biotin-labeled targets (5 μg/array) at 60°C for 17 hours. Filters were first washed with 2 × SSC/1% SDS and then with 0.1× SSC/1% SDS at 60°C for 15 minutes each. Chemiluminescent detection steps were performed by subsequent incubation of the filters with alkaline phosphatase–conjugated streptavidin and CDP-Star substrate. The images were captured using FluorChem 8900 (Alpha Innotech Corporation, San Leandro, CA, http://www.alphainnotech.com).

For data analysis, the positive and negative spots were independently identified and verified by at least two people. Only matched positive and negative results from two independent experiments were used for analysis. For quantification, intensity of signal was first measured using ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, CA, http://www.mdy.com) with a local background subtraction method. These subtracted intensities were then divided by the average of intensities from glyceraldehyde-3-phosphate dehydrogenase (GAPD) (three spots in each array) to obtain a relative intensity for each spot. These relative intensities were used to calculate correlation value (R^2) related to input amounts of cRNAs or RNAs or to compare samples.

RESULTS

Developing a Human Dopaminergic and Glial Focused Array

A total of 281 known genes were chosen to construct a human dopaminergic neuronal and glial array (Table 1). Among these genes, 127 represented surface markers, receptors, transporters, and transcription factors that are characteristic of dopaminergic neurons, astrocytes, or oligodendrocytes. Another 36 genes were a subset of genes highly expressed in pluripotent cells and neural progenitors. The remaining approximately 100 genes included cytokines and their receptors, transcription factors, and extracellular matrix molecules that have been known to play important roles in neural development. This focused array should in principle be able to examine dopaminergic neuronal and glial development and also monitor the contamination of pluriopotent and neural progenitors from which these cells are typically derived.

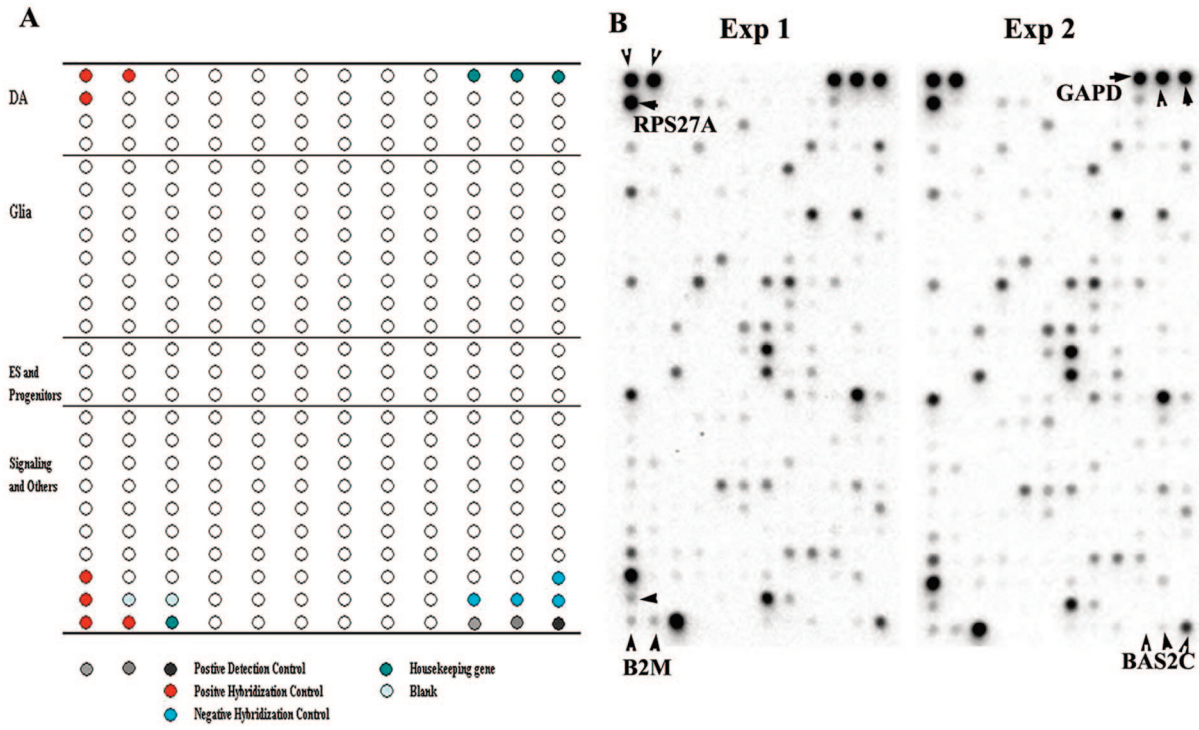
The gene specific 60-mer 3′ biased oligonucleotides for these 281 genes were designed and arrayed in a 25 × 12 format. These oligo probes were grouped and printed based on their function (Fig. 1A). Housekeeping genes, such as *GAPD*, *β-actin*, *RPS27A*, and *B2M*, served as positive controls. *GAPD* was spotted in triplicate in the upper right corner, and hybridization intensities were used as a reference to normalize data. *RPS27A* and *B2M*, known to be expressed high and low, respectively, were spotted on the top and bottom of the left side and served as additional positive hybridization controls. Biotinylated artificial sequence 2 complementary (BAS2C) sequences were spotted at an increasing gradient on the right corner to serve as a positive detection control. Negative controls, such as a blank, plasmid PUC18 DNA, or artificial sequences not expected to be present in cDNA, were also included as detection and background hybridization controls. This array format allows us to easily monitor quality of array hybridization.

Quality Control Testing

The quality of the array was tested by first examining printing efficiency using tracking dye (0.001% phenol red) and that was followed by control hybridization tests. Tracking dye was evenly and uniformly distributed on all printed spots (data not

Table 2. A list of primer sequences

Gene name	Forward sequence	Backward sequence
DDC	GGGACCACAACATGCTGCTC	CCACTCCATTCAGAAGGTGCC
DAT	CTGGTG TCTGGAAGATCTGC	AGCTGTCTCCACTGGAGTCA
TH	GGTTCCCAAGAAAAGTGTCAG	GGTGTAGACCTCCTTCCAG
Smo	TATTCACTCCCGCACCAAC	AGCCAGACATCCAGAACTC
GFRA1	AGGGAAATGATCTGCTGGAGGA	CTCTGGCTGGCAGTTGGTAAAA
VMAT2	ACACAAAATGGGGAGGTGG	AGCAGAGAGGGGCAAAAAG
VACHAT	ACGTGGATGAAGCATACG	ACGTGGATGAAGCATACG
GFAP	TGGTAGAGATGGAGGAGGAG	GTATGACACAGCAAGGAAGAG
Olig1	CACCTTTCGTTTCCCTTTCC	GCTACTACCAACAACCAAAACC
Olig2	TGCTCCTCTTCCTCCTTTC	AACCCCCAAATAACCCAAAC
S100β	AGGGGTGAGACAAGGAAGAG	ATAGCAGAAAGAATGATGCAGG
Nanog	CAAAGGCAAACAACCCACTT	TCTGCTGGAGGCTGAGGTAT
Oct3/4	CTTGCTGCAGAAGTGGGTGGAGGAA	CTGCAGTGTGGGTTTCGGGCA
DPPa5	ATGGGAACTCTCCCGGCACG	TCACTTCATCCAAGGGCCTA
Podxl	TCTTACCCCTCCCTACACTC	CAGCCACTGCTCTTTCATAC
Prom1	ATGACAAGCCCATCACAAC	TAAAGCACTACCCAGAGACC
G3PDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGGCCATGAGGTCCACCAC



C

Groups	Gene Name	Exp 1 Mean	Exp 1 SD	Exp 1 CV %	Exp 2 Mean	Exp 2 SD	Exp 2 CV %
Housekeeping	GAPD	1	0.04	4	1	0.07	7
Positive Hyb Ctrls	RPS27A	0.99	0.09	9	0.99	0.06	6.1
	B2M	0.2	0.04	14	0.19	0.08	12
Whole Chip		0.11	0.25		0.12	0.25	

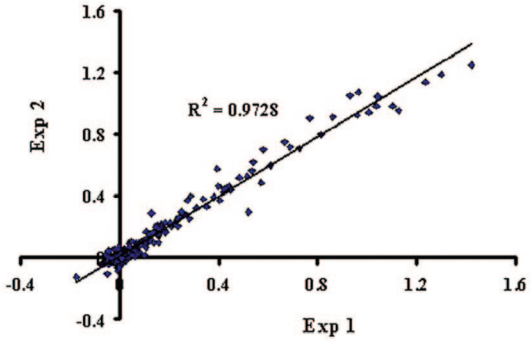


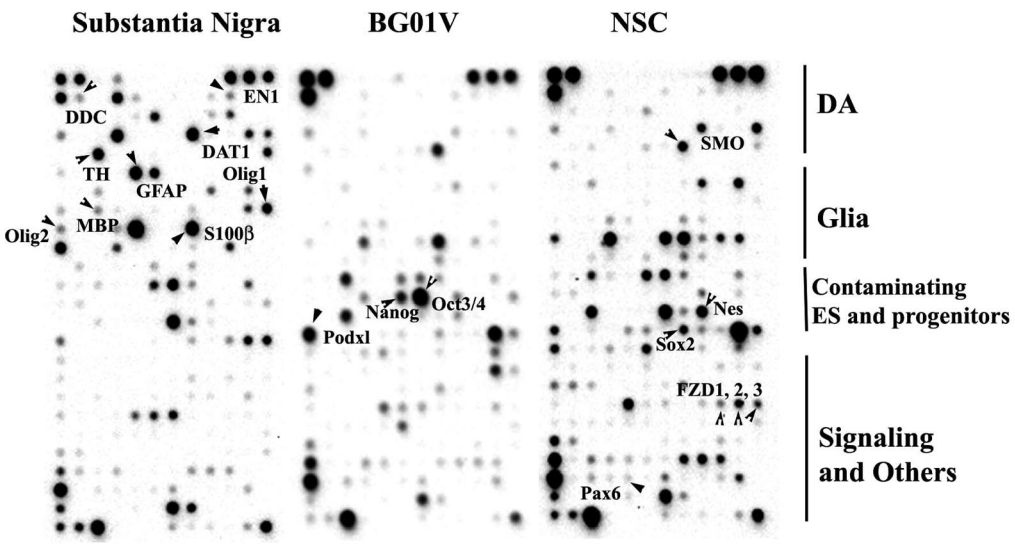
Figure 1. Array format and quality control. **(A):** Array layout and gene distribution based on gene functions. **(B):** Hybridization image profile of two experiments. BAS2C sequences were printed in an increasing gradient fashion to serve as a positive detection control. The arrows indicate triplicates of positive hybridization controls and housekeeping genes. The spot intensity was measured and normalized to GAPD. **(C):** The average (mean) and SD for triplicates are summarized. The CV value was calculated with the formula SD divided by mean and was expressed as a percentage. **(D):** A linear correlation plot of these two experiments with an R^2 value of 0.97. Abbreviations: BAS2C, biotinylated artificial sequence 2 complementary; CV, coefficient of variance; GAPD, glyceraldehyde-3-phosphate dehydrogenase.

shown). All spots were visually present in all array membranes. For the array hybridization test, we first generated biotin-labeled cRNA targets with the following sources of RNA: BG03, BG03-derived NSCs, differentiated NTera2 cells, and human caudate brain (Clontech). Then these biotinylated cRNAs were equally mixed (7 μg/sample) and used to hybridize to the focused arrays (5 μg/membrane). Image profiles of duplicate experiments are shown in Figure 1B. As expected, the BAS2C spots showed a gradient intensity increase, all positive controls showed positive spots, and there were no spots in any negative controls for blanks, plasmid, and artificial sequences. Quantification of triplicates of positive controls showed a small coefficient of variance (<15%; Fig. 1C). Duplicate experiments showed a high correlation as well, with an R^2 value of 0.98, indicating excellent reproducibility (Fig. 1D). In summary, the array testing indicated that the oligonucleotide probes were printed evenly, appropriately, and attached to the membrane tightly. The hybridization process did not permit significant cross-hybridization, and expression levels were within the dynamic range of the controls.

Validation Testing

Next, we examined the ability of focused array to selectively detect genes highly expressed in dopaminergic neurons, glial, and embryonic stem, and neural progenitors by using cell-

A



B

Groups	# genes	Gene Name
DAnergic Markers (Nigra$^+$BG01V$^-$NSC$^-$)	9	AADC(DDC); DRD1IP(CALCYON); DRD2; EN1; MAOA; MAOB; SLC6A3(DAT1); SNCB; TH.
Glial Markers (Nigra$^+$BG01V$^-$NSC$^-$)	18	CST3; GFAP; GJB1; KCNJ10; MBP; MOG; NKX2-2; NKX6-2; OLIG1; OLIG2; PDGFRA; UGT8; ID4; S100B; ASAH1; SLC1A1; MCOLN1; PLP1.
Contaminating ES and Progenitors (Nigra$^-$BG01V$^+$NSC$^+$)	13	CER1; NANOG; POU5F1(Oct3/4); PODXL; TERF1; ABCG2; ACTA2; NES; SOX2; POU3F2; PROM1; SLC2A1; SOX3.

C

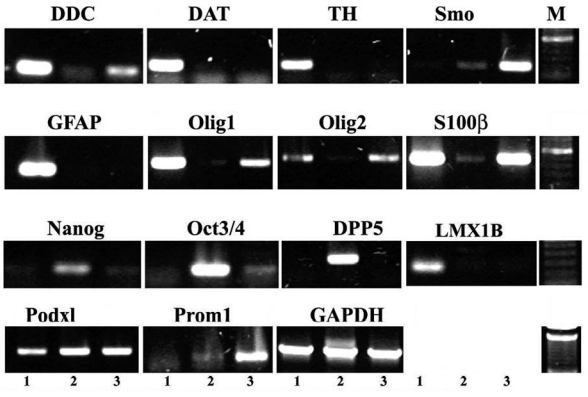


Figure 2. Validation experiments. The RNA from human substantia nigra, BG01V, and BG03-derived NSCs were first converted into cDNA and then transcribed to cRNAs in the presence of biotin-16-UTP. These biotinylated cRNAs (5 μg/membrane) were hybridized to the focus array at 60°C for 17 hours. **(A):** Hybridization profiles. Functional gene groups are indicated on the right. **(B):** The tissue-specific genes identified in each group are summarized. **(C):** RT-PCR confirmation. Substantia nigra (lane 1), BG01V (lane 2), and NSC (lane 3). Abbreviations: DA, dopaminergic neurons; NSC, neural stem cell; RT-PCR, reverse transcription–polymerase chain reaction.

specific samples. We chose to use adult human substantia nigra as a source of material that would include dopaminergic neurons and glial cells, and the hESC line BG01V and NSCs derived from the hESC line BG03 as samples that would contain pluripotent and progenitor cells.

The patterns of gene expression profiles easily distinguished these three cell types (Fig. 2A). In the human substantia nigra sample, the array detected high gene expression of dopaminergic and glial markers with no or low expression of pluripotent markers. In undifferentiated BG01V and NSCs, the array showed high expression levels of pluripotent markers and little expression of dopaminergic and glial markers. Correlation analysis among these samples with normalized intensities indicated that the adult substantia nigra sample poorly correlated with BG01V ($R^2 = 0.50$) and NSC ($R^2 = 0.59$) samples, whereas BG01V correlated well with NSC ($R^2 = 0.81$) samples. We detected nine dopaminergic neuron markers and 18 glial markers in the adult substantia nigra samples; however, few of these markers were detected in the BG01V and NSC samples. For example, tyrosine hydroxylase (TH), dopa decarboxylase (DDC), and dopamine transport (DAT), enzymes required for either synthesis or transport of the neurotransmitter dopamine in

dopaminergic neurons, were highly detected in the adult substantia nigra (Fig. 2A, arrows) but not observed in BG01V and NSC samples. Likewise, glial fibrillary acidic protein (GFAP), S100-β, myelin basic protein, and oligodendrocyte transcription factor 1 and 2 (Olig1 and Olig2), all known glial markers for either astrocytes or oligodendrocytes, were also detected in the adult substantia nigra but not observed in BG01V and NSC samples. Pluripotent markers, including Nanog and Oct3/4, were detected in BG01V samples and not detected in the adult substantia nigra samples. Sox2 and nestin, markers typically expressed in NSCs or progenitors, were highly expressed in NSC samples but low or absent in the adult substantia nigra. These tissue-specific genes detected in the array are summarized in Figure 2B. Additionally, some of these tissue-specific genes were used to perform RT-PCR analysis to confirm array data (Fig. 2C). Thus, this array can distinguish dopaminergic neurons and glial cells from ESC and NSC populations.

The array profiles also show differential expression patterns of signaling molecules among BG01V, NSCs, and adult substantia niagra samples. For example, Frizzled receptors, including members 1, 2, and 3, were detected in NSCs but absent or expressed at low levels in BG01V and substantia nigra (Fig. 2A, arrows). The array results show that PAX6 was highly expressed in NSC but not in BG01V, and expression in the substantia nigra sample was quite low. Thus, the array may also provide useful information that will allow us to dissect signaling changes that occur during neural development.

Although numerous markers and signaling molecules were detected in a tissue-specific fashion, we noted that some genes, which are known to be expressed in these tissues, were not detected by this array. For example, *LMX1B*, a transcriptional factor promoting dopaminergic neuron development, and *Dpp5a*, an hESC-specific pluripotent marker, were both detected by RT-PCR (Fig. 2C) but not detected by the array. This is likely caused by low sensitivity of the array compared with RT-PCR and highlights the importance of direct testing and redesigning of these probes further to improve array quality.

Titration Testing

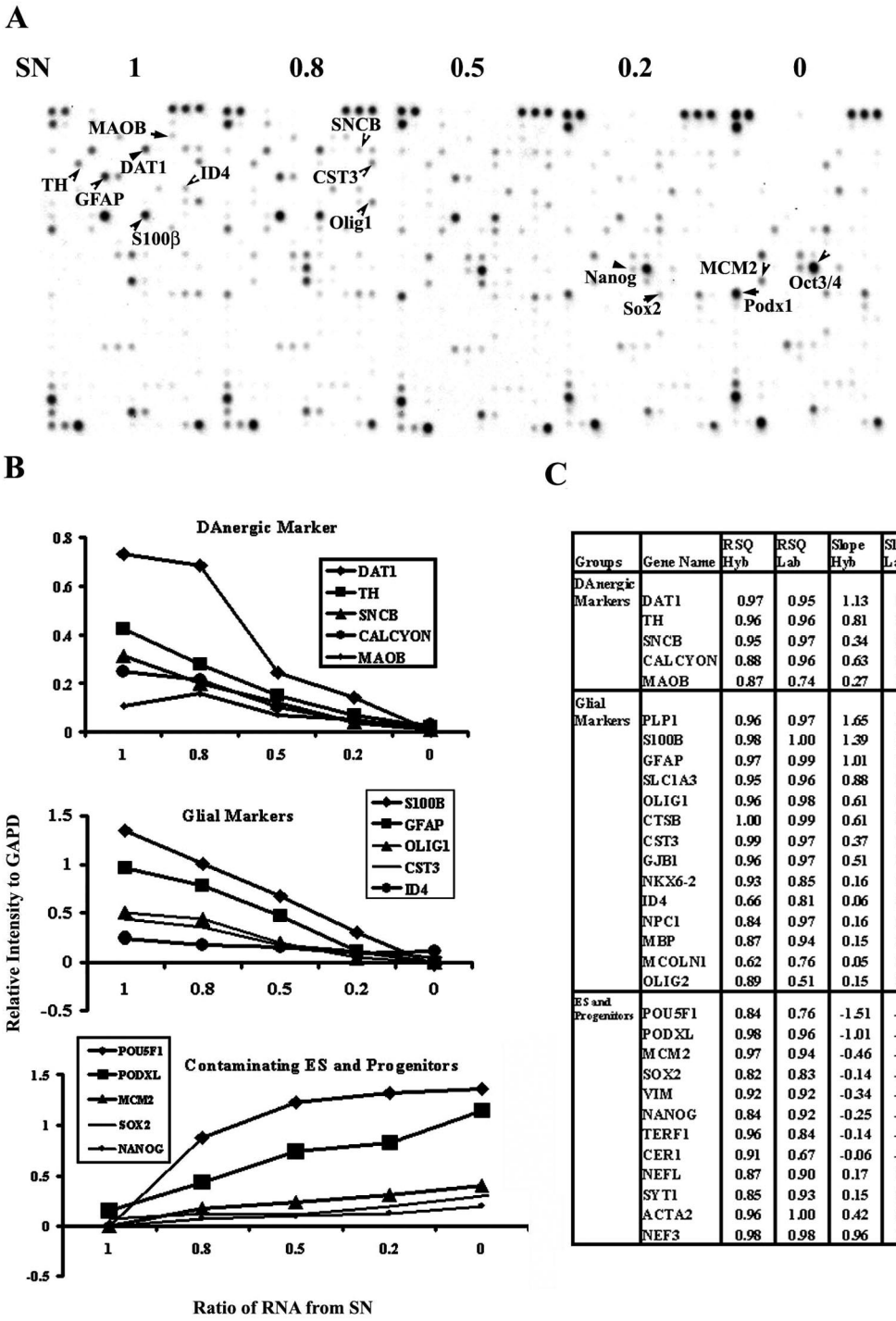
Given the ability of the array to distinguish dopaminergic neurons, glia, and hESCs, we next performed titration experiments to determine whether the array could detect tissue-specific genes in a concentration-dependent manner. These experiments included titration of both the hybridization and labeling processes. For titration experiments in testing hybridization, we first generated biotionylated cRNA targets from human substantia nigra and/or hESC line BG01V. Then, we held the total cRNA (5 μg/array) constant and altered the ratio of cRNA from human substantia nigra to hESC BG01V as follows: 1:0, 0.8:0.2, 0.5:0.5, 0.2:0.8, and 0:1. The hybridizations were performed, and intensity data were normalized to the housekeeping gene *GAPD*. The results showed a positive linear relationship of detected hybridization signals of the above tissue-specific genes to inputs of cRNAs with R^2 values from 0.6 to 0.99 (RSQHyb in Fig. 3C), indicating that the hybridization condition was optimal. Next, we tested whether the amplification labeling system was linear to total RNA input (2 μg/labeling) by using the same ratio of human substantia nigra to human BG01V samples as those in hybridization experiments. Visually, intensity changes of specific genes are correlated to their input amounts of RNA, such as DAT1, TH, GFAP, and Oct3/4 (Fig. 3A). As shown in Figure 3B, there was a high correlation between detected signals of cell type–specific genes and RNA inputs. The R^2 values and slopes obtained from both titration experiments in hybridization alone and hybridization plus labeling process are summarized in Figure 3C. The results showed that the labeling and hybridization system was optimal to detect these cell-specific markers in a dose-dependent manner. Considering these data, we suggest that these dose-response tissue-specific genes may serve to develop a reporting list of candidate differentially expressed genes for detection of dopaminergic neurons and glial cells in mixed populations.

Application Testing

Next, we examined the process of neuronal differentiation using this array. Previously, we have used hESC line BG01 and BG02 to induce dopaminergic differentiation by co-culture with the mouse stromal cell line PA6 [14]. Because these hESCs lines are routinely maintained on MEFs and need a special culture protocol to avoid differentiation, we have recently used human teratocarcinoma–derived embryonal carcinoma cell line NTera2, which does not need a feeder layer for maintenance and can be differentiated into functional dopaminergic neurons using the same PA6 co-culture inducing protocol [13]. Comparing global gene expression between NTera2 and multiple undifferentiated hESCs shows a similarity in sharing multiple stem cell markers. NTera2 differentiation by PA6 co-culture resembles hESCs in marker expression, efficiency, and time course, suggesting that Ntera2 can serve as a surrogate for hESCs [13]. We have shown by both of fluorescence-activated cell sorting and immunostaining analysis that PA6 cells do not express TH, PSA-NCAM, and SSEA-4. PSA-NCAM and TH expression increases as Ntera2 cells differentiated on PA6 cells, whereas the expression of the pluripotency marker SSEA-4 decreases after PA6 induction [13]. Here, NTera2 cells were first induced toward the dopaminergic lineage by co-cultured with mouse stromal cell line PA6 for 12 days. To enrich for potential neuronal cells, these NTera2-PA6 co-cultures were then sorted by flow cytometry for PSA-NCAM. For comparison purposes, we also included undifferentiated NTera2 cells and sorted PSA-NCAM$^-$A2B5$^+$ (FA2B5$^+$) cells from human brain at 20 weeks of gestation. Total RNAs from these cells were isolated and used for hybridization experiments.

The array results indicated that the sorted PSA-NCAM$^+$ NTera2 cells expressed markers for dopaminergic neurons. The hybridization image profile of the sorted PSA-NCAM$^+$ NTera2 cells (Fig. 4A) showed more hybridized spots in the dopaminergic marker group (first five rows of array) than did those of undifferentiated NTera2 and FA2B5$^+$ populations. Dopaminergic markers, such as TH and DDC, showed higher expression in the PSA-NCAM$^+$ NTera2 samples than in either the undifferentiated NTera2 or the FA2B5$^+$ samples (Fig. 4B). Engrailed 1, a transcriptional factor, and Smoh, a receptor for Shh, were also expressed in the PSA-NCAM$^+$ NTera2 sample. RT-PCR and immunostaining results further verified the array results (Fig. 4C, 4D, first row).

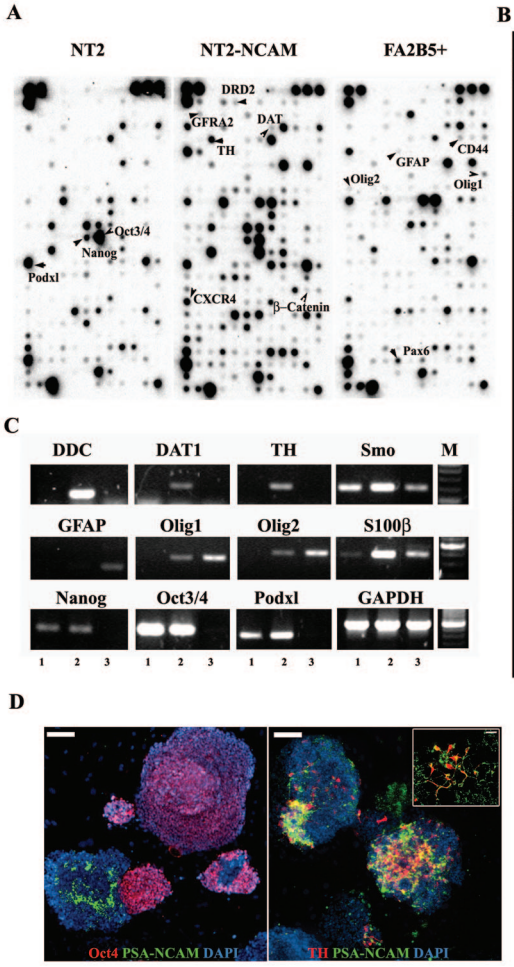
However, the array also showed that the sorted PSA-NCAM$^+$ NTera2 cells contained pluripotent and neural progenitor cells (Fig. 4). Pluripotency markers, such as Oct3/4, Nanog, and Podxl, were found in both PSA-NCAM$^+$ NTera2 cells and undifferentiated NTera2 cells. This was further confirmed by



Groups	Gene Name	RSQ Hyb	RSQ Lab	Slope Hyb	Slope Lab
DAnergic Markers	DAT1	0.97	0.95	1.13	0.78
	TH	0.96	0.96	0.81	0.39
	SNCB	0.95	0.97	0.34	0.30
	CALCYON	0.88	0.96	0.63	0.23
	MAOB	0.87	0.74	0.27	0.11
Glial Markers	PLP1	0.96	0.97	1.65	1.61
	S100B	0.98	1.00	1.39	1.32
	GFAP	0.97	0.99	1.01	1.01
	SLC1A3	0.95	0.96	0.88	0.67
	OLIG1	0.96	0.98	0.61	0.55
	CTSB	1.00	0.99	0.61	0.55
	CST3	0.99	0.97	0.37	0.41
	GJB1	0.96	0.97	0.51	0.39
	NKX6-2	0.93	0.85	0.16	0.14
	ID4	0.66	0.81	0.06	0.13
	NPC1	0.84	0.97	0.16	0.11
	MBP	0.87	0.94	0.15	0.11
	MCOLN1	0.62	0.76	0.05	0.06
	OLIG2	0.89	0.51	0.15	0.04
ES and Progenitors	POU5F1	0.84	0.76	-1.51	-1.20
	PODXL	0.98	0.96	-1.01	-0.90
	MCM2	0.97	0.94	-0.46	-0.35
	SOX2	0.82	0.83	-0.14	-0.20
	VIM	0.92	0.92	-0.34	-0.19
	NANOG	0.84	0.92	-0.25	-0.17
	TERF1	0.96	0.84	-0.14	-0.12
	CER1	0.91	0.67	-0.06	-0.03
	NEFL	0.87	0.90	0.17	0.09
	SYT1	0.85	0.93	0.15	0.12
	ACTA2	0.96	1.00	0.42	0.31
	NEF3	0.98	0.98	0.96	1.01

Figure 3. Titration experiments. These experiments were performed in both hybridization and labeling processes. The total cRNA targets were kept at a constant value (5 μg/array). The ratio of human SN to human ES BG01V was kept the same either in cRNA targets of the hybridization experiment or total RNA in the labeling process as follows: 1:0, 0.8:0.2, 0.5:0.5, 0.2:0.8, and 0:1. **(A):** Images obtained from the labeling experiments. The values on the row above the image indicate ratio of input of total RNA from human SN. Some markers that changed their intensities along with inputs are indicated on the images. **(B):** Plots of quantified relative intensity versus RNA inputs. **(C):** The linear correlation efficiency (RSQ) and slope between relative intensities and amounts of inputs is summarized. RSQHyb or SlopeHyb: derived from the hybridization experiments; RSQLab or SlopeLab: obtained from the labeling processes. Abbreviations: ES, embryonic stem; RSQ, R^2; SN, substantia nigra.

RT-PCR (Fig. 4C). To test whether these pluripotent markers were really expressed in the PSA-NCAM$^+$ NTera2 cells or represented contaminating cell populations, we performed co-localization studies using PSA-NCAM and Oct4 antibodies. As shown in Figure 4D, PSA-NCAM was not co-localized with Oct4, indicating a contamination of undifferentiated NTera2



B

Groups	Gene name	NT2	NT2-NCAM	FA2B5+
DAnergic Markers	SMOH	0.14	0.65	0.06
	TH	0.00	0.37	0.01
	SULT1A1	0.14	0.33	0.17
	CALCYON	0.02	0.15	0.03
	DRD2	-0.02	0.07	0.00
	DDC	-0.01	0.05	-0.06
	Engrailed 1	-0.01	0.03	0.00
	MAOB	0.00	0.01	-0.01
Glial Markers	ID2	0.08	1.20	0.84
	SOX4	0.09	1.15	1.41
	ID4	0.03	1.05	0.56
	Krox-24	-0.01	1.00	0.51
	Survivin	0.15	0.95	0.10
	CSTB	0.26	0.92	0.27
	CTSC	0.17	0.47	0.03
	PLP1	-0.01	0.16	0.00
	NPC1	0.03	0.13	0.06
	MCOLN1	0.00	0.07	0.02
	Cystatin C	0.00	0.06	0.04
	ASAH1	0.01	0.04	0.01
	GFAP	0.00	0.02	0.00
	SLC1A1	0.01	0.02	0.01
	OLIG1	0.01	0.02	0.09
	S100B	0.01	0.02	-0.03
	OLIG2	0.00	0.01	0.01
	MBP	-0.01	0.00	0.01
	MOG	0.00	0.00	-0.01
ES and Progenitors	POU5F1	1.01	1.29	-0.01
	PODXL	0.65	0.84	0.06
	VIM	0.43	1.35	1.04
	MCM2	0.28	1.25	0.21
	NEF3	0.06	1.45	0.11
	TERF1	0.04	0.15	0.07
	NANOG	0.04	0.35	0.00
	ACTA2	0.03	0.13	0.02
	SOX2	0.02	0.67	0.24
	Cerberus	0.01	0.00	0.01
	NES	-0.03	0.48	0.11

Figure 4. Application experiments. The cRNA targets (5 μg/array) derived from undifferentiatied NTera2 cells (NT2), NTera2 cells induced toward dopaminergic neurons by co-culture with mouse stromal cell line PA6 for 12 days followed by flow cytometry enrichment for PSA-NCAM expressing cells (NT2-NCAM), and $NCAM^{-}A2B5^{+}$ (labeled as $FA2B5^{+}$) cells from human brain at embryonic 20 weeks ($FA2B5^{+}$) were hybridized to the array at 60°C for 17 hours. **(A):** The hybridization images of those cells. **(B):** The relative intensity to GAPD of the reporting genes was calculated and is summarized. **(C):** RT-PCR confirmation. NT2 (lane 1), NT2-NCAM (lane 2), and $FA2B5^{+}$ (lane 3). **(D):** Co-localization immunocytochemistry staining of NTera2 AP6 co-cultures at day 12. The cells were immunostained live with anti–PSA-NCAM, fixed, and stained with either Oct4 or TH. Bars = 200 μm, (inset) 50 μm. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; GAPD, glyceraldehyde-3-phosphate dehydrogenase; PSA, polysialic acid; RT-PCR, reverse transcription–polymerase chain reaction; TH, tyrosine hydroxylase.

cells in the sorting processes. Sox2 and nestin, markers expressed in NSCs and progenitor cells, were expressed at higher levels in $NCAM^{+}$ NTera2 cells than in the $A2B5^{+}$ glial progenitor populations. The $NCAM^{+}$ NTera2 cells and $A2B5^{+}$ population shared the expression of potential glial lineage markers olig1, olig2, ID2, and cystatin C but lacked expression of more mature phenotypic markers, astrocytes and oligodendrocytes. Thus, the array results provided useful information for characterization of these cell types and show that the array can be used to detect contamination of a partially purified population.

DISCUSSION

Our results show that the dopaminergic-glial focused array that we have developed can reliably distinguish between undifferentiated cells and their progeny differentiating along the neural lineage, and is sufficiently sensitive to detect as little as 10% contamination from pluripotent cells or progenitors. We can further identify lineage-specific genes that are likely candidates for developing future quantitative PCR–based arrays. Additionally, this array allows the identification of signaling pathways involved in the process of differentiation and suggests that, as additional data accumulate, critical shared regulatory mechanisms will be identified.

Several important parameters were assessed to ensure that the produced array be reliable and reproducible. We used sophisticated selection criteria to identify oligonucleotides of the same length which showed minimal cross-hybridization and had roughly the same annealing temperatures, allowing for an increased sensitivity and reduced background. We tested the uniformity of loading and arraying using labeled oligonucleotides and measuring overall spot intensity. Additionally, hybridization with single probes showed that each probe identified its cognate partner and not another gene on the array (data not shown). Loading controls at three different concentrations measured saturation and provided a rough measure of the relative level of gene expression. For additional control purposes, we included random oligomers as negative controls and included oligonucleotides on the array, such that spiking with probes would allow one to assess the quality of labeling and hybridization. These controls serve to provide immediate feedback on the hybridization experiment and on whether one can compare between two independent experiments.

It is useful to emphasize the advantage of being able to format the array such that visual information can be provided even before a detailed analysis is performed. We have previously shown that results are identical when probes are randomly

placed or organized into useful subsets [9]. In these experiments, we separated potential markers for pluripotent and progenitor cells, oligodendrocytes, astrocytes, and dopaminergic neurons and placed cytokines and their receptors in separate groups (see Results). Visually, it was clear simply from the pattern of gene expression which cell type was being analyzed. This provides a quick feedback prior to a detailed quantitative analysis and the relative levels of expression (compared with the controls spotted at different concentrations) and allows one to more carefully adjust the scanning parameters for more quantitative studies.

We used human substanatia niagra samples to assess the sensitivity and reliability of our arrays. Human substanatia niagra was chosen because it contains a subset of dopaminergic neurons in a mixed population and thus represents a common situation in the laboratory. Examining gene expression identified markers, such as TH, DDC, and DAT (see Results), in which the magnitude of change was sufficient to be readily assessed. These results were further confirmed by RT-PCR using standardized primer sets we developed for all genes present on the array. Testing a variety of samples showed that this focused array could reliably distinguish dopaminergic neurons and glial cells from ESCs and NSCs. The lack of any mismatch of cell type–specific markers by the array indicated the absence of problems in probe design, printing contamination, or cross-hybridization, and these were key criteria in establishing the validity of the focused array. Moreover, our titration experiments, in which we gradually mixed the substantia nigra sample with the hESC sample, showed a linear relationship of hybridization intensity with input RNA, further supporting the reliability of this array. The titration experiments were very helpful in identifying genes that distinguish one cell type from another on the focused array.

Although we very carefully chose a list of genes for dopaminergic neurons and glial cells based on the published literature, not all of genes were differentially expressed at levels that could be detected by the array. This is likely due to their expression levels and sensitivity of the array hybridization process as their expression could be confirmed by RT-PCR (data not shown). For example, LMX1B, a transcription factor required for dopaminergic neuronal development, was detected by RT-PCR in human substantia nigra; however, the array could not detect LMX1B in the human substania niagra sample. Although one could redesign the probes and further optimize hybridization, it would be impractical to regenerate new lists with increased sensitivity given that this array was sufficiently sensitive to distinguish populations and monitor dopaminergic differentiation. The responses of reporting genes in titration experiments were concentration-dependent in both the hybridization and labeling processes with high linear correlation efficiency (0.7–1). The slopes of their response to inputs reflected the sensitivities of detection in this array. However, we generated a more restricted list of genes that shows the largest range of difference; we suggest that these be used to develop a more sensitive quantitative PCR assay if such an assay is required.

In addition, we included an additional 118 genes that are known to play essential roles in neural development which could assist in dissecting molecular events occurring during neuronal development. These include signaling molecules for Wnt-Fzd, TGF-β, Notch, fibroblast growth factor (FGF), and BMP (bone morphogenetic protein) pathways. By comparison of gene expression profiling in signaling groups between undifferentiated NTera2 cells and PA6-induced sorted cells, we have noted that some known and required pathways for induction and formation of dopaminergic neurons were activated in the $NCAM^+$ NTera2 sorted population. We observed enhanced expression of Smoh, Nr4a2, En1, GFRA2, and Fzds in the sorted differentiating cells that have previously been reported as signaling pathways in dopaminergic differentiation. Shh-Smoh activation and FGF8 signaling are known to be key players in midbrain pattering and genesis of dopaminergic neurons [15, 16]. Nr4a2 is a transcription factor and is expressed in both dopaminergic precursors and neurons in ventral midbrain, and deletion of Nr4a2 results in a loss of dopaminergic neurons in ventral midbrain [17–19]. Engrailed genes (*En1* and *En2*) were shown to be involved in dopaminergic neuron survival and maintenance [20]. Activation of Wnt-Fzds pathways has multiple functions, including promoting proliferation of NSCs and dopaminergic precursors and differentiation from dopaminergic precursors to their mature neurons depending on the members of Wnts involved [21–23]. Although further dissection of signaling pathways involved in promoting dopaminergic formation of NTera2 cells is required, our results suggest that similar pathways are activated in PA6-induced dopaminergic differentiation and in midbrain dopaminergic neuron formation during development.

Many have debated the utility of focused arrays versus a global array, which contains all genes present on a focused array and provides substantial additional information. In the past, we have argued that most large-scale arrays are not complete and often do not contain the genes that are of immediate relevance. Although still true, newer whole genome arrays from providers such a NimbleGen Systems, Inc. (Madison, WI, http://www.nimblegen.com), Illumina (San Diego, http://www.illumina.com), and Affymetrix (Santa Clara, CA, http://www.affymetrix.com) are closer to ensuring the presence of all genes of interest. Similarly, as technology has advanced, global arrays have become less expensive than previous arrays. A large array, although 10 times more expensive ($500–$600 [USD]), incurs costs in reagents and personnel time similar to a focused array and provides more information. Nevertheless, we feel that a focused array offers several advantages. A focused array is still significantly cheaper to develop and run, and it can be run easily by any laboratory. Also, the data analysis and reporting issues are easily manageable without elaborate bioinformatics support. It is potentially useful for some screening when a number of samples have to be treated for a short time. Posting and comparing data across laboratories is also relatively simple given that criteria of sensitivity, cutoffs, and hybridization efficiency are much easier to address for a few genes than for larger numbers of genes. The sensitivity of assessment using selected genes is often higher than in global arrays, in which statistical methods and large numbers of replicates have to be run to extract meaningful information [24, 25]. Adding new genes (e.g., markers for other types of neurons, such as GABA-type), rearranging formats, and adding additional controls is trivial in a focused array, which allows the maintenance of continuity with old data sets.

In summary, our goal was to develop a list of genes that could be used effectively in a focused array format for routine assessment of the process of differentiation. We desired an

inexpensive method that was robust and reliable and could be used on a routine basis to monitor differentiation. Our results showed that a focused array fulfills these criteria and permits the monitoring of at least 300 genes (an order of magnitude larger than before) as cells differentiate. Careful selection of the genes has permitted one to distinguish stages of differentiation and degree of contamination of undifferentiated cells and identify the crucial signaling pathways that direct the process of differentiation. As data accumulate with different populations and different methods of differentiation, one will perhaps be able to identify the key regulators and biomarkers that may allow further reduction of the number of genes needed to monitor specific populations of neural derivatives.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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